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PATENT APPLICATION
Docket No. UMMC91-03A

GP 1813

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



Applicants: Harriet L. Robinson, Ellen F. Fynan, and Robert G. Webster

Serial No.: 08/009,833 Group Art Unit: 1813

Filed: January 27, 1993 Examiner: L. Smith

Title: IMMUNIZATION BY INOCULATION OF DNA TRANSCRIPTION UNIT

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Washington, D.C. 20231 on March 8, 1994
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

Barbara A. Frank 3-8-94
Signature Date

DECLARATION OF HARRIET L. ROBINSON, PH.D.

The Honorable Commissioner
of Patents and Trademarks
Washington, DC 20231

Sir:

Transmitted herewith is an executed Declaration of Harriet L. Robinson, Ph.D. The unexecuted Declaration was

filed with an Amendment in the Patent Office on February 22, 1994.

Respectfully submitted,

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Dated: March 8, 1994

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Hamilton, Brook, Smith & Reynolds, P.C.

Barbara A. Forant

Signature

Barbara A. Forant

Typed or Printed Name of Person Signing Certificate

DECLARATION OF HARRIET L. ROBINSON

The Honorable Commissioner
of Patents and Trademarks
Washington, DC 20231

Sir:

I, Harriet L. Robinson, of 3 Birchwood Avenue, Southboro, MA 01772, hereby declare and state that:

1. I am a co-inventor on the above-identified patent application.

2. The following experiments were conducted by me or under my supervision.

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3. Studies on pCMV/H1 DNA immunization in a ferret model were undertaken because this influenza model has many similarities to human influenza infections. In the initial experiment, ferrets were immunized with purified pCMV/H1 DNA in saline by intramuscular inoculations at a one month interval. Young adult female ferrets were prebled and vaccinated with 500 μg of pCMV/H1 or pCMV/control DNA in saline by two injections of 125 μl in each hind leg for a total inoculation volume of 500 μl. One ferret received three intramuscular inoculations of 500 μg of pCMV/H1 DNA at one month intervals while a second animal received two intramuscular inoculations of 500 μg of DNA at one month intervals. The control animal received three 500 μg intramuscular inoculations of pCMV/control DNA at one month intervals.

4. Metofane-anesthetized ferrets were challenged with $10^{7.7}$ egg infectious $doses_{50}$ of A/PR/8/34 (H1N1) via the nares at one week after the final DNA inoculation. Nasal washes were collected at days 3, 5 and 7 post challenge under ketamine anesthetic. Titration of virus in nasal washes was done in eggs as described (Katz, J.M. and R.G. Webster, *J. Infect. Dis. 160*: 191-198 (1989)). Data are presented in Table 1, below.

TABLE 1: Protection of Ferrets against an H1 Virus by Intramuscular Inoculation of pCMV/H1 DNA

| DNA | No. of DNA Adminis-trations | Ferret ID No. | Virus Titer in Nasal Washes, $log_{10}$ egg infectious $doses_{50}$/ml | | |
|---|---|---|---|---|---|
| | | | day 3 | day 5 | day 7 |
| pCMV/H1 | 3 | 901 | 5.5 | 1.5 | <1 |
| | 2 | 903 | 5.7 | 4.7 | <1 |
| pCMV/control | 3 | 907 | 6.5 | 6.2 | <1 |

5. Analyses of nasal washes revealed similar high titers of virus in the washes of all of the ferrets at 3 days post challenge. Interestingly, the ferret receiving three inoculations of pCMV/H1 had largely cleared the nasal infection by five days post challenge, with its five day nasal wash containing less than 10 egg infectious $doses_{50}$ of virus per ml. At this time the ferret receiving two inoculations of pCMV/H1 DNA had a ten fold reduction in the titer of virus in its nasal wash. By contrast, the ferret receiving control DNA had modest if any reduction in the titer of virus in its nasal wash. By 7 days post challenge, all of the ferrets had cleared their nasal infections. The much more rapid clearing of virus in the ferret receiving three intramuscular inoculations of pCMV/H1 DNA and the somewhat more rapid clearing of virus in the ferret receiving two intramuscular inoculations of pCMV/H1 DNA than in the two ferrets receiving control DNA suggest that the intramuscular inoculations of pCMV/H1 had raised some anti-influenza immunity.

6. To increase the efficiency of the induction of immunity, a second experiment was undertaken in

ferrets using the Accell gene gun to deliver DNA coated gold beads into the skin of ferrets. The abdominal epidermis was used as the target for gene gun delivered DNA with ferrets receiving two gene gun administrations of DNA at a one month interval. Gene gun inoculations were delivered to Ketamine-anesthetized young adult female ferrets. Skin was prepared by shaving and treating with the depilatory agent NAIR (Carter-Wallace, New York). DNA beads (1 to 3 microns) were prepared for inoculations as previously described (Fynan *et al.*, *Proc. Natl. Acad. Sci. USA 90*:11478-11482 (1993)). A delivery voltage of 15 kV was used for inoculations. Ferrets were inoculated with either 2 μg or 0.4 μg of DNA. Ferrets inoculated with 2 μg of DNA received 10 shots with each shot consisting of 0.8 mg of beads coated with 0.2 μg of DNA. Ferrets receiving 0.4 μg of DNA received two of these shots.

7. Metofane-anesthetized ferrets were challenged at one week after the second DNA immunization by administration of $10^{6.7}$ egg infectious doses of A/PR/8/34 (H1N1) virus via the nares. This challenge was 10 fold lower than in the experiment using intramuscular inoculation because of the high levels of virus replication in the first challenge. Nasal washes were collected at days 3 and 5 post challenge under ketamine anesthetic and the virus titered as described above. Data are presented in Table 2, below.

Table 2: Protection of Ferrets against an H1 Virus by Gene Gun Inoculation of pCMV/H1 DNA

| DNA | Amount of DNA ($\mu$g) | Ferret ID No. | Virus Titer in Nasal Washes, $\log_{10}$ egg infectious $doses_{50}$/ml | |
|---|---|---|---|---|
| | | | day 3 | day 5 |
| pCMV/H1 | 2 | 927 | <1 | <1 |
| | | 931 | <1 | <1 |
| | | 933 | <1 | <1 |
| | 0.4 | 926 | 4.3 | <1 |
| | | 929 | 3.9 | <1 |
| | | 933 | <1 | <1 |
| pCMV/control | 2 | 932 | 3.5 | <1 |
| | | 934 | 3.7 | <1 |

8. Analyses of post-challenge nasal washes in gene gun vaccinated ferrets revealed that the three ferrets receiving 2 $\mu$g of DNA and one of the three ferrets receiving 0.4 $\mu$g of DNA were completely protected from the challenge. This was shown by the inability to recover virus in the nasal washes of these animals at 3 days post challenge. The remaining two animals receiving 0.4 $\mu$g of DNA and the control animals were not protected, with easily detected titers of virus present in the nasal washes of the animal at three days post challenge. In this experiment, all animals (control and vaccinated) had no detectable virus in their nasal washes by five days post challenge.

9. Ferrets from the gene gun experiment were next analyzed for antibody responses to the DNA administrations and to the challenge virus. These assays tested for neutralizing activity for A/PR/8/34 (H1N1). The titrations of antibodies were done as

described (Katz, J.M. and R.G. Webster, *J. Infect. Dis. 160*: 191-198 (1989)). Titers of neutralizing activity are the reciprocals of the highest dilution of sera giving complete neutralization of 200 50% tissue culture infectious doses of virus. Data are presented in Table 3, below.

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Table 3: Neutralizing Antibody in Ferrets Vaccinated with Gene Gun-Delivered pCMV/H1 DNA and Challenged with A/Pr/8/34 (H1N1) Influenza Virus

| DNA | Amount of DNA (μg) | Ferret ID No. | Neutralizing Antibody | | | |
|---|---|---|---|---|---|---|
| | | | Pre-inoculation | Post-boost, pre-challenge | Post challenge (7 days) | Post challenge (14 days) |
| pCMV/H1 | 2 | 927 | <10 | <10 | 2500 | 1800 |
| | | 931 | <10 | 800 | 2500 | 1800 |
| | | 933 | <10 | 130 | 4000 | 4000 |
| | 0.4 | 926 | <10 | <10 | 25000 | 25000 |
| | | 929 | <10 | <10 | 4000 | 1300 |
| | | 933 | <10 | <10 | 7900 | 5600 |
| pCMV/control | 2 | 932 | <10 | <10 | 5600 | 4000 |
| | | 934 | <10 | <10 | 5600 | 7900 |

16. Neutralizing antibody post DNA boost but prior to challenge was detected in two of the animals receiving 2 μg of gene gun-delivered DNA. No neutralizing antibody was detected in the pre-challenge sera of the third animal receiving 2 μg of DNA (an animal that was completely protected against the presence of virus in nasal washes). Neutralizing antibody was also not detected in the sera of the ferret receiving 0.4 μg of DNA that did not develop virus in its nasal wash.

17. In animals with prechallenge antibody, protection was presumably due to the presence of neutralizing antibody as well as the mobilization of memory responses for neutralizing antibody. In protected animals without detectable levels of prechallenge antibody, protection was likely due to the rapid mobilization of memory responses by the infection, with the mobilized responses controlling the infection.

I further declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Harriet L. Robinson

3/1/94
Date

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